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Phylogenetic Relationships of *Isoëtes* (Isoëtaceae) in China as Revealed by Nucleotide Sequences of the Nuclear Ribosomal ITS Region and the Second Intron of a *LEAFY* Homolog

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Abstract.—*Isoëtes* is an ancient lycopod lineage with a highly conserved morphology that provides few morphological characters to resolve the phylogeny of its species. Species appear to have evolved by divergence and allopolyploidy. The basic diploids *I. hypsophila*, *I. taiwanensis*, and *I. yunguiensis* and the tetraploid *I. sinensis* occur in China. Analysis of ITS sequences indicates that the Chinese *Isoëtes* species are part of an Australasian clade including *I. brevicula* from Western Australia and *I. kirkii* from New Zealand. Two distinct cloned sequences of the second intron of a *LEAFY* homolog were recovered from *I. sinensis* supporting the hypothesis that *I. sinensis* is an allotetraploid. One of the *I. sinensis* cloned sequences was similar to the *I. taiwanensis* sequence and the other cloned sequence was similar to the *I. yunguiensis* sequence identifying *I. taiwanensis* and *I. yunguiensis* as the likely parents of *I. sinensis*. Other cloned sequences recovered from *I. sinensis* were recombined parts of the two distinct sequences. Morphological evidence supporting an allotetraploid origin of *I. sinensis* is found in its larger microspore size and intermediate megaspore texture compared to *I. taiwanensis*, and *I. yunguiensis*.

Isoëtes L. is a cosmopolitan genus of heterosporous lycopods containing hundreds of species. Plants usually appear as tufts of linear leaves arising from an underground, corm-like rootstock. Ellipsoidal sporangia occur in expanded leaf bases. Species range from evergreen aquatics to ephemeral terrestrials. Although *Isoëtes* is an ancient lineage with its distinctive morphology recognizable in the Triassic (Retallack, 1997), few characters have been found in its highly conserved morphology to resolve the phylogenetic relationships of its species. Distinguished by their habitat preference, megaspore morphology, and chromosome numbers, species appear to have evolved by ecological isolation and genetic divergence as separated populations adapted to terrestrial or aquatic habitats and by interspecific hybridization and chromosome doubling (allopolyploidy) when divergent species were dispersed into the same sites (Taylor et al., 1993).

Interspecific hybridization and allopolyploidy are well documented for *Isoëtes*. Many interspecific hybrids have been recognized by their production of irregular spores and confirmed by their chromosome numbers (Brunton and

Britton, 1999). In several cases, interspecific hybrids and their suspected allopolyploid derivatives have been verified by chromosome counts, isozyme profiles, and DNA sequences (Taylor and Hickey, 1992; Hoot and Taylor, 2001; Hoot et al., 2004). A polyploid series ranging from $3x = 33$ to $12x = 132$ is known for *Isoëtes*. Over 60% of *Isoëtes* taxa, for which chromosome counts have been published, are polyploid (Troìa, 2001). Therefore, not only is there documentation that interspecific hybridization and allopolyploidy occur in *Isoëtes*, but there is also evidence that allopolyploidy is an equally important mechanism of speciation in this genus.

Recently, herbarium, field, and laboratory studies have been conducted to learn more about the *Isoëtes* of China. These studies have provided an opportunity to determine the status of historical populations, discover new populations and new taxa, and obtain live specimens from which root tips could be harvested for chromosome counts and fresh leaves could be collected for DNA isolations.

Four species of *Isoëtes* have been described for China. All are believed to be rare and endangered. *Isoëtes hypsophila* Handel-Mazzetti is known from the Hengduan Mountains in northwestern Yunnan Province and southwestern Sichuan Province. In this region, *I. hypsophila* occurs in the shallow water of lakes and ponds about 3600 m above sea level. *Isoëtes sinensis* T. C. Palmer has been found at about ten sites in and along rivers and lakes of the middle and lower Yangtze River system. At present, only three populations are known to remain in China. *Isoëtes sinensis* has also been reported from the Kyushu and Chubu Districts Japan (Takamiya et al., 1997) and Cheju Island, South Korea (Takamiya, 2001). *Isoëtes taiwanensis* DeVol is known only from Menghuan Lake near the foot of Zhixing Mountain in the Yangming Mountains National Park, north of Taipei in northern Taiwan. *Isoëtes yunguiensis* Q. F. Wang and W. C. Taylor is known from the Yunnan–Guizhou Plateau in southwest China. In this region, plants have been recorded at four sites, but only two small populations, totaling about 400 individuals, are known to remain. Liu et al. (2002) reported that *I. hypsophila*, *I. taiwanensis*, and *I. yunguiensis* are basic diploids ($2n = 2x = 22$) and *Isoëtes sinensis* is a tetraploid ($2n = 4x = 44$).

Nucleotide sequences from the nuclear ribosomal ITS region, the chloroplast *atpB-rbcL* spacer region, and the second intron of a *LEAFY* homolog have been used to determine phylogenic relationships of *Isoëtes*, delimit species, and reveal an interspecific hybrid and its allotetraploid derivative (Hoot and Taylor, 2001). Hoot *et al.* (2004) used cloning to separate homoeologous sequences of the second intron of a *LEAFY* homolog for several *Isoëtes* allotetraploids. By comparing these cloned sequences to those of putative parents, some of the parent species could be identified.

The goals of this paper were to use nucleotide sequences from the nuclear ribosomal ITS region and the second intron of a *LEAFY* homolog to: (1) determine the relationships of the Chinese *Isoëtes* species, (2) test the hypothesis that the tetraploid *I. sinensis* is an allotetraploid and, if this hypothesis is correct, (3) identify the basic diploid parent species of *I. sinensis*.

TABLE 1. Specimens sampled. Columns indicate species, location, collector, collection number-DNA isolation number, date of collection, and herbarium acronym for location of voucher. Collections are from Mainland China unless otherwise noted.

Species	Voucher Collection	Isolation Number
Isoëtes brevicula	Rock pool, summit of Lily McCarthy Rock, Western Australia, 25 Sep 2002, *W. C. Taylor & N. T. Luebke 6383* (MIL)	189
Isoëtes hypsophila	Tu-er-sjan, Dao-cheng County, Sichuan Province, 01 Aug 2001, *Wang Aing-Feng, Liu Xing, Liu Hong & Yang Xiao-Lin 2* (WH)	127
Isoëtes kirkii	Lake Brunner, South Island, New Zealand, 27 Mar 2004, *D. W. Woodland & Felicity Cutten s.n.* (MIL)	*kiNZ*
Isoëtes sinensis	Xing-an-jiang, Jiande City, Zhejiang Province, 19 Oct 2001, *Liu Xing & Pang Xin-An 3, 4* (WH)	129, 131
Isoëtes taiwanensis	Menghuan Lake, Yangming Mountains, Taiwan, May 1998, *Chiou Wen-Liang s.n.* (MIL)	78
Isoëtes yunguiensis	Sha-shi-chong, Ping-ba County, Guizhou Province, 15 Aug 2001, *Liu Xing & Yang Xiao-Lin 5* (WH)	130

MATERIALS AND METHODS

Species sampling.—Table 1 contains locality, collector, collection number, and location of voucher specimens for plants used in this study. Specimens were identified to species using the original descriptions of the species (Handel-Mazzetti, 1923; Palmer, 1927; DeVol 1972a; Wang Q. F. *et al.*, 2002) and by comparison with authentic and type specimens. Diagnostic morphology for megaspore textures was evaluated using an Olympus SZX12 stereomicroscope.

DNA isolation and amplification.—DNA was isolated from 20 mg of silica dried leaves from each sample by grinding the leaf tissue, frozen in liquid nitrogen, to a powder with a disposable 1.5 pellet pestle (Kimble-Kontes) in a 1.5 ml snap-cap microcentrifuge tube (Eppendorf) and using the DNeasy® Plant Mini Kit (Qiagen) following the manufacturer's protocol.

The ITS region for all samples was amplified with the primers ITS-I (5′-GTCCACTGAACCTTATCATTTAG-3′; Urbatsch, *et al.* 2000) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.* 1990). The *LEAFY* intron for all samples was amplified with the primers 30F (5′- GATCTTTATGAA-CAATGTGG-3′) and 1190R (5′- GAAATACCTGATTTGTAACC-3′); Nancy S. Napier designed both *LEAFY* primers. PCR reaction mixtures followed manufacturer protocols using Ready-To-Go™ PCR Beads (Amersham Bio-sciences). PCR amplification for the ITS region began with denaturation for 60 s at 97°C followed by 40 cycles of denaturation for 10 s at 97°C, annealing for 30 s at 48°C, and extension for 20 s at 72°C with 4 s added to extension time each cycle and ending with a final extension of 7 min at 72°C. PCR amplification for the *LEAFY* intron began with denaturation for 5 min at 94°C followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C, and ending with a final extension of 5

min at 72°C. PCR products were concentrated via electrophoresis in a 2% agarose gel containing ethidium bromide and visualized with transilluminated UV. Bands were cut from the gel and purified using the QIAquick® Gel Extraction Kit (Qiagen).

Cloning and sequencing.—Sequencing of the ITS PCR products was performed on both 5′ and 3′ DNA strands using the amplification primers ITS-I and ITS4 as cited above.

Ligation of the purified *LEAFY* PCR product and subsequent transformation, cloning, and visualization of transformed clones followed manufacturer protocols using the pGEM®-T Easy Vector Systems (Promega) with LB Amp 100 X-gal plates (Teknova).

For basic diploid species, *I. taiwanensis* and *I. yunguiensis*, eight clones (colonies) for each species were sequenced. For the tetraploid species, *I. sinensis*, 16 clones (eight from each of two plants) were sequenced to increase the odds of capturing all parental cloned sequences. For the outgroup species, *I. brevicula* E. R. L. Johnson, *I. hypsophila*, and *I. kirkii* A. Braun, four clones from each species were sequenced.

Cleaning and concentration of the vector DNA followed manufacturer protocols using the QIAPrep® Spin Miniprep Kit (Qiagen). Sequencing was performed on both 5′ and 3′ DNA strands using sequencing primers M13F and M13R in conjunction with the ABI Big Dye® Terminator Cycle v 3.1 Sequencing Kit (Applied Biosystems) following the manufacturer's protocol. Sequencing products were resolved on an ABI (model 377) DNA sequencer at the Iowa State University DNA Sequencing and Synthesis Facility, Ames, Iowa.

Sequence alignment and phylogenetic analysis.—Nucleotide sequences were aligned and edited using Sequencher 4.1 (Gene Codes Corp.). Gaps were treated as additional presence/absence characters, with one or multiple base gaps scored as a single character (Baldwin *et al.* 1995).

Maximum parsimony analysis of the data was conducted with PAUP* version 4.0b10 (Swofford, 2002) using the heuristic search option for the ITS data set and the *LEAFY* data set, maximum trees = 4000 for the ITS data set and maximum trees = 100 for the *LEAFY* data set. PAUP* was used to run 500 bootstrap replicates for each data set to estimate reliability of the clades (Felsenstein, 1995).

RESULTS

ITS sequences of *Isoëtes hypsophila*, *I. taiwanensis* and *I. yunguiensis* from China, *I. brevicula* from southwestern Australia, and *I. kirkii* from New Zealand form an Australasian clade with other species and clades previously reported by Hoot and Taylor (2001). The data set analyzed consisted 16 ingroup species and two outgroup species with a total of 826 characters; 313 characters were variable and 239 were parsimony informative. The ITS tree illustrated is a bootstrap 50% majority-rule consensus tree of the 39 most parsimonious trees retained (Fig. 1). Tree topology shows a close relationship

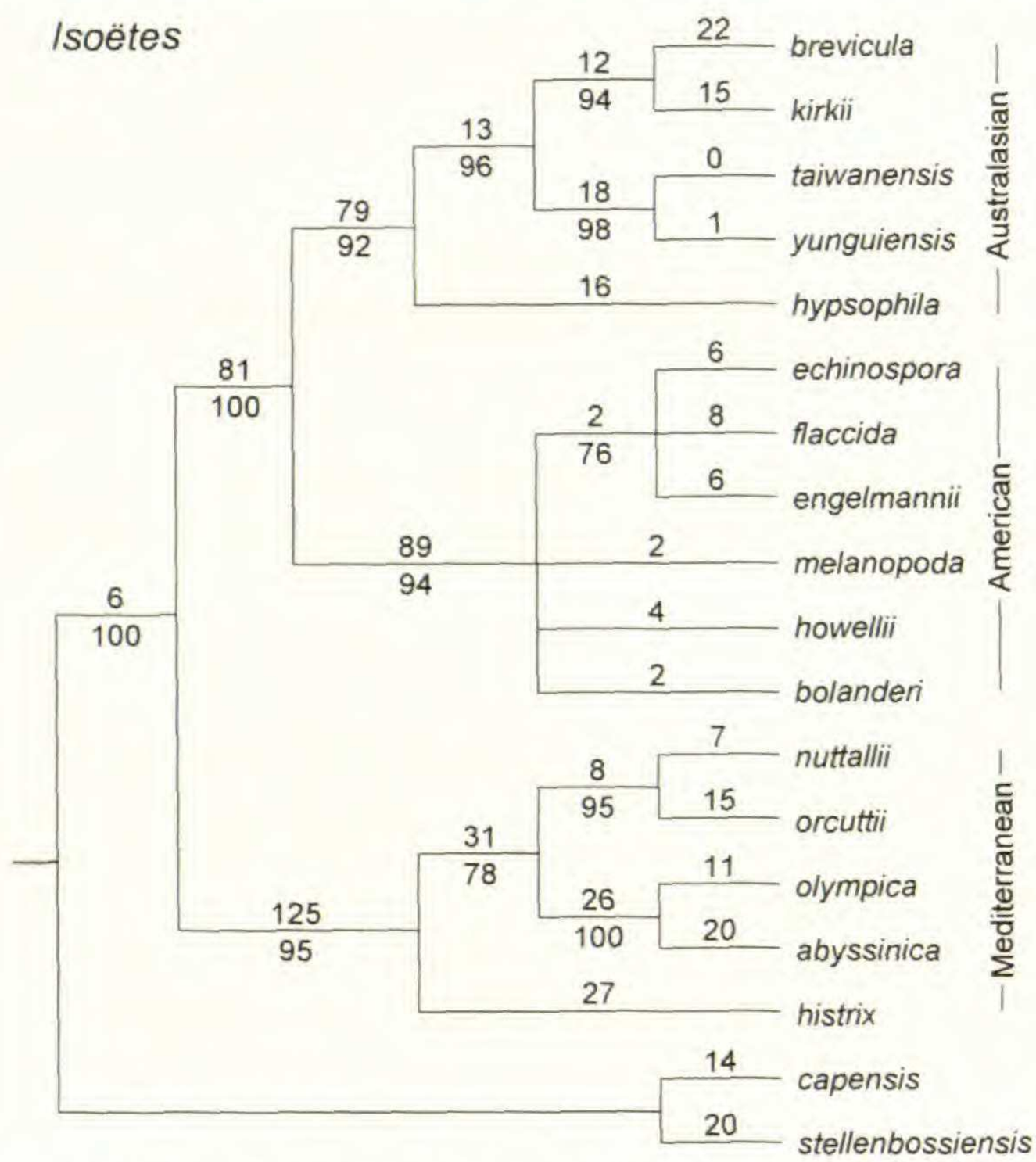

FIG. 1. *Isoëtes* ITS region tree. Bootstrap 50% majority-rule consensus tree of 39 trees resulting from maximum parsimony analysis using heuristic search of ITS region sequence data for eighteen basic diploid species of *Isoëtes*. Tree length is 489 steps of equally weighted nucleotide substitutions and gaps, CI (excluding uninformative characters) = 0.76, RI = 0.86. Numbers above the branches are the number of nucleotide substitutions. Numbers below the branches are bootstrap values. Major clades are labeled on the right. The tree is rooted with *I. capensis* and *I. stellenbossiensis* from South Africa.

exists between *I. taiwanensis*, and *I. yunguiensis*. All species in the tree are basic diploids ($2n = 22$).

Defined by the 30F and 1190R Napier primers, the *LEAFY* region for aligned sequence clones of *I. sinensis*, *I. taiwanensis*, and *I. yunguiensis* was 1105 bases long and included all of the second intron and parts of the flanking exons. All of the eight sequence clones of *I. taiwanensis* were 1075 bases long and all of the eight sequence clones of *I. yunguiensis* were 1072 bases long. Two of the 16 *I. sinensis* clones did not amplify for the *LEAFY* intron. Therefore, 14 aligned, cloned sequences were compared for informative sites. At 15 informative sites that included 12 substitutions, two one base gaps, and one three base gap, six cloned sequences of *I. sinensis*, each 1076 bases long, matched or nearly matched the cloned sequence type of *I. taiwanensis* and five cloned sequences of *I. sinensis*, each 1079 bases long, matched or nearly matched the consensus cloned sequence type of *I. yunguiensis* (Table 2). Seven *I. sinensis* cloned sequences showed evidence of recombination i.e., part of the PCR amplified, cloned sequence first matched either the *I. taiwanensis* or the *I. yunguiensis* sequence type, but further on matched the other sequence type.

TABLE 2. Comparison of fourteen cloned sequences of *I. sinensis* with cloned sequences of *I. taiwanensis* and *I. yunguiensis* at fifteen informative sites. Sites are numbered sequentially beginning with the 30F Napier primer. *Isoëtes sinensis* cloned sequences are identified by collection number-DNA isolation number-clone number. Nucleotides in italic compare to those of *I. taiwanensis*. Nucleotides in bold face compare to those of *I. yunguiensis*. Gaps are indicated by dashes. Sequences marked with an asterisk showed extensive recombination and were removed from the data set before the final analysis. Clones 4-131-6 and 4-131-7 did not amplify for the *LEAFY* region and are not included in the table.

site/clone	205	289	290	357	525	549	660	741	776	827	840	865	965-967	987	1059
taiwanensis	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
3-129-3	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
3-129-7	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-1	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-3	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-4	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
3-129-5	*C*	*G*	*T*	*T*	*C*	*G*	*T*	**C**	*A*	*A*	*A*	*A*	- - -	-	**G**
3-129-2*	*C*	*G*	*T*	*T*	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	- - -	-	*A*
3-129-1*	*C*	*G*	*T*	*T*	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	*A*
4-131-8*	**T**	**A**	**C**	**C**	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-2	*C*	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	-	**G**
3-129-4	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	-	**G**
3-129-8	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	-	**G**
3-129-6	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	**G**
4-131-5	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	**G**
yunguiensis	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	**G**

Three sequences showing evidence of extensive recombination were removed from the data set evaluated by PAUP*.

Based on the relationships indicated in the ITS tree (Fig. 1), *I. brevicula*, *I. kirkii*, and *I. hypsophila* were chosen as outgroup species to root the *LEAFY* second intron tree. The sequenced *LEAFY* region for *I. brevicula* was 1035 bases, for *I. kirkii* it was 1090 bases, and for *I. hypsophila* it was 1095 bases.

The *LEAFY* second intron data set analyzed contained 16 sequences with a total of 1125 characters; 171 characters were variable and 54 were parsimony informative. The data set included consensus sequences from clones of *I. brevicula*, *I. hypsophila*, *I. kirkii*, *I. taiwanensis*, and *I. yunguiensis* and 11 cloned sequences of *I. sinensis*. The *LEAFY* second intron tree illustrated is a bootstrap 50% majority-rule consensus tree of the two most parsimonious trees retained (Fig. 2). Six of the *I. sinensis* cloned sequences formed a clade with *I. taiwanensis* and five of the *I. sinensis* cloned sequences formed a clade with *I. yunguiensis*.

DISCUSSION

Although *Isoëtes* is Paleozoic in origin (Pigg, 1992), worldwide in distribution, and over time, undoubtedly adapted to changing climates and aquatic to terrestrial habitats on every continent many times, the morphology of *Isoëtes* has been remarkably conserved. Thus, morphology provides few characters that can be used to reliably reconstruct phylogenetic relationships. Nevertheless, pteridologists have speculated about the relationships of *Isoëtes*

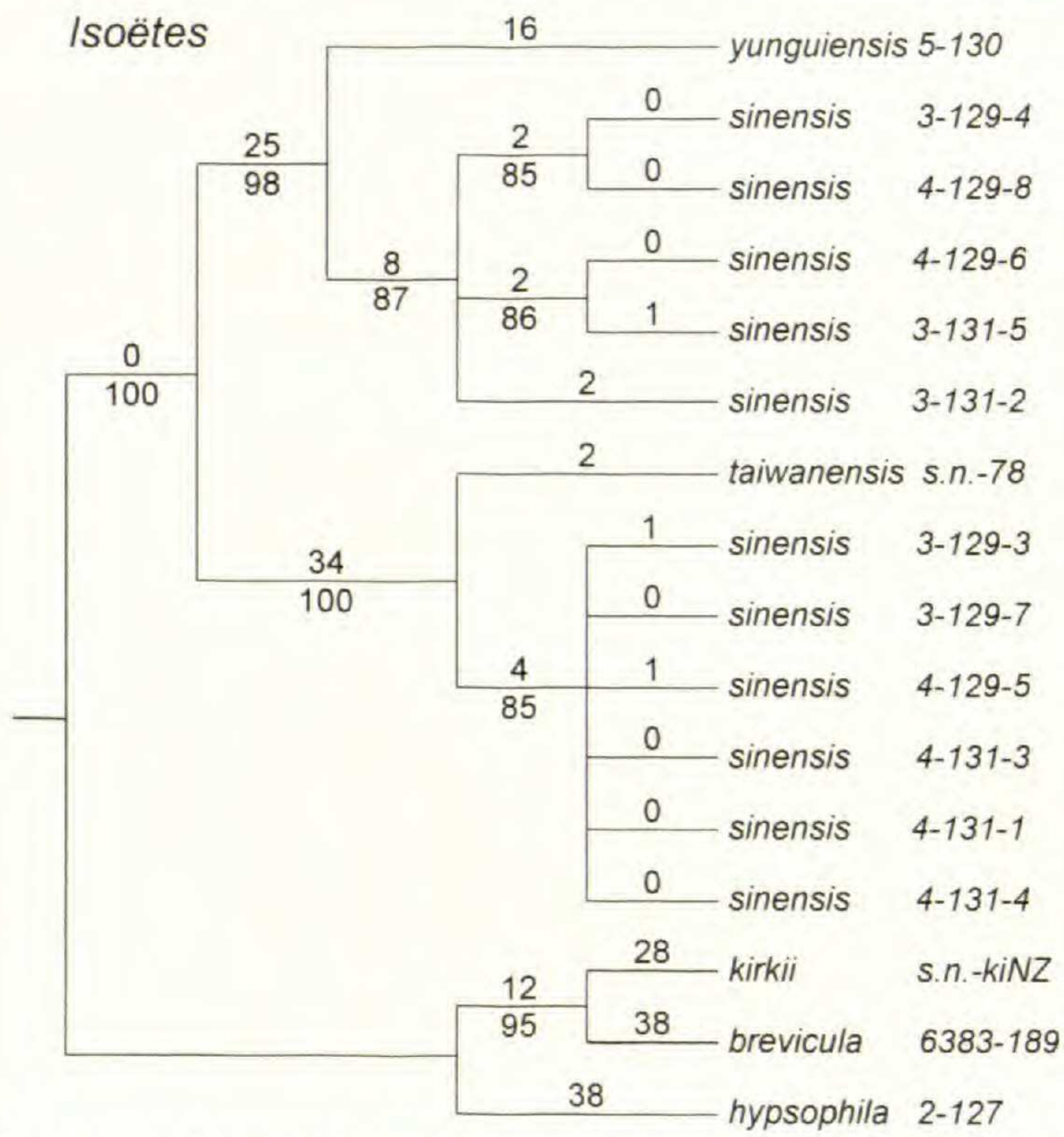

FIG. 2. *Isoëtes LEAFY* second intron homolog tree. Bootstrap 50% majority-rule consensus tree of four trees resulting from maximum parsimony analysis using heuristic search of *LEAFY* second intron homolog sequence data for five basic diploid and one tetraploid (*I. sinensis*) species of *Isoetes*. Eleven sequence clones that form a sister clade to either *I. yunguiensis* or *I. taiwanensis* represent the tetraploid genome of *I. sinensis*. Tree length is 183 steps of equally weighted nucleotide substitutions and gaps, CI (excluding uninformative characters) = 0.89, RI = 0.96. Numbers above the branches are the number of nucleotide substitutions. Numbers below the branches are bootstrap values. Figures to the right of the specific epithets are the collection and clone identification labels. The tree is rooted with *I. hypsophila* from China, *I. brevicula* from Western Australia, and *I. kirkii* from New Zealand.

species based on ecology, morphology, and biogeography. Britton and Brunton (1991) reevaluated the spore morphology of *I. taiwanensis*, concluding that it was not related to taxa from southwestern Australia as proposed by Marsden (1979), but instead appeared to have its closest affinity to *I. kirkii* from New Zealand. The ITS tree (Fig. 1) shows that both *I. brevicula* from southwestern Australia and *I. kirkii* form a sister clade to the Chinese species and all are members of an Australasian clade. Based on spore morphology, habit, and habitat, Huang *et al.* (1992) concluded that *I. taiwanensis* is probably closer to *I. asiatica* than it is to *I. sinensis*, but *I. asiatica* (*I. echinospora* subsp. *asiatica* (Makino) Á. Löve is a member of the *I. echinospora* species complex, a group of circumpolar taxa with echinate megaspores (Löve, 1962, Takamiya, 1997). The ITS tree (Fig. 2) shows that *I. echinospora*, a member of an American clade, is only distantly related to members of the Australasian clade, which includes *I. taiwanensis*. DeVol (1972b) mentioned that *I. taiwanensis* seemed nearer to *I. sinensis* than any other species. Takamiya (2001) saw similarities in the spore morphology of *I. taiwanensis* and *I. sinensis* and concluded that phylogenetic comparisons of these two taxa were needed.

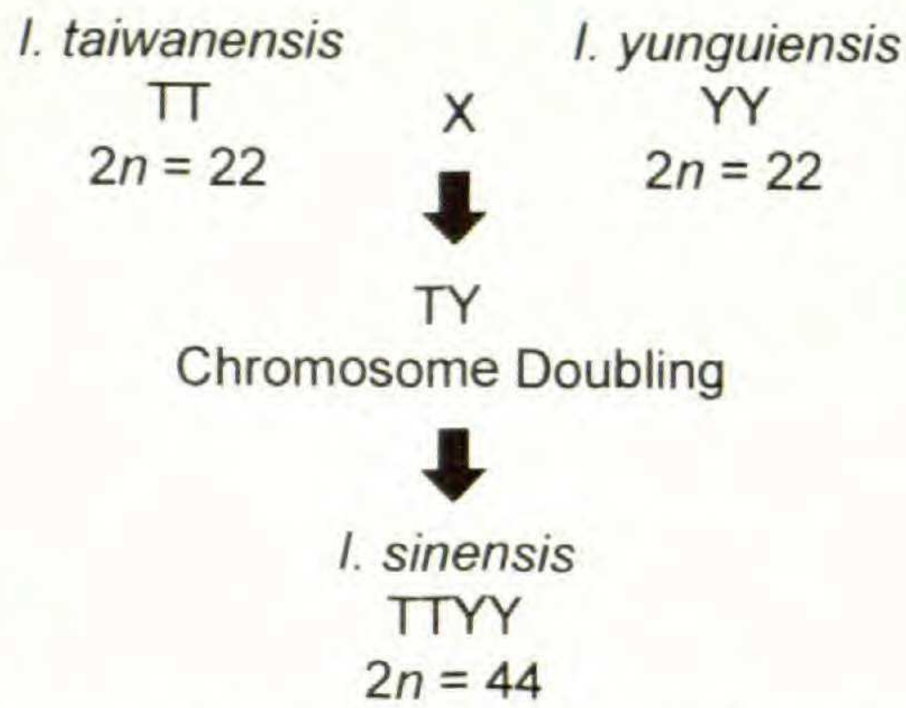

FIG. 3. Hypothetical phylogeny of *Isoëtes sinensis* involving interspecific hybridization and chromosome doubling of the basic diploids *I. taiwanensis* and *I. yunguiensis*. Data presented in this study supports the hypothesis of an allotetraploid origin for *I. sinensis*.

The recovery of two, distinct, *LEAFY* second intron sequence types from the tetraploid *I. sinensis* supports the hypothesis that *I. sinensis* is an allotetraploid (Table 2). To clarify the results, it was assumed that some of the sequenced clones recovered from *I. sinensis* were recombinations of the two distinct sequence types and three of the recombinant sequences were removed from the data set evaluated by PAUP*. Since recombination occurs from crossing over between chromosomes during meiosis, it is possible that the observed recombined sequences were products of natural events. If the recombined sequences detected were the result of crossing-over during meiosis, we would predict that identical crossover sequences would be recovered as clones. All seven of the recombinant cloned sequences from *I. sinensis* were different, indicating that these recombined sequences more likely occurred during the PCR amplification reaction. Whatever their source, including recombinant sequences in a cladistic analysis will affect results and therefore, they need to be recognized and removed from the data set before the final analysis.

Comparison of the two distinct cloned sequences from the allotetraploid *I. sinensis* with the cloned sequences of *I. taiwanensis* and *I. yunguiensis* indicates that either these two basic diploid species, or closely related taxa, likely participated in the formation of *I. sinensis* (Table 2). Although the *I. yunguiensis* clade, including five *I. sinensis* cloned sequences, and the *I. taiwanensis* clade, including six *I. sinensis* cloned sequences, are both well supported with high bootstrap percentages, the *I. yunguiensis* sequence is distinguished from its sister *I. sinensis* clones by sixteen autapomorphies and the *I. taiwanensis* sequence is distinguished from its sister *I. sinensis* clones by two autapomorphies (Fig. 2). These unique nucleotide substitutions could be due to (1) sequencing nucleotides of taxa different from those of the parent taxa, (2) continued evolution of the progenitor parent species and the allotetraploid species following allopolyploidy, or (3) copy errors during PCR. Causes for the autapomorphies might be determined by additional sampling and repeated PCR of the same clones.

In addition to the molecular characters, morphological characters indicate that *I. sinensis*, *I. taiwanensis*, and *I. yunguiensis* are distinct, but closely

related species and provide some evidence supporting the allopolyploid origin of *I. sinensis*. All three species are amphibious plants with tri-lobed rootstocks. They all have a rudimentary velum that covers only the upper edge of the sporangium. The microspores of *I. taiwanensis* and *I. yunguiensis* range from 20–26 μm in length whereas, those of *I. sinensis* range from 26–30 μm in length (Britton and Brunton, 1991; Wang *et al.*, 2002; Palmer, 1927). The larger size of *I. sinensis* microspores is attributed to its increased chromosome number. Increases in chromosome number are usually accompanied by larger spore size (Kott and Britton, 1983). In contrast, megaspores of the tetraploid *I. sinensis* and the basic diploid *I. yunguiensis* average about 400 μm in diameter, whereas megaspores of the basic diploid *I. taiwanensis* average about 300 μm in diameter. Megaspore texture appears to be the most distinctive character that separates these three species. However, in view of the results presented here, the cristate to verrucate megaspores of *I. sinensis* can also be interpreted as subtly combining the textures of the rugulate to reticulate megaspores of *I. taiwanensis* and the cristate to reticulate megaspores of *I. yunguiensis*.

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APPENDIX

Genbank accession numbers for the new *Isoëtes* DNA sequences used in this manuscript.

Species	Genbank accession number
Nuclear ribosomal ITS region sequences	
I. brevicula	AY641098
I. hypsophila	AY641099
I. kirkii	AY641100
I. taiwanensis	AY641101
I. yunguiensis	AY641102
LEAFY second intron homolog sequences	
I. brevicula	AY641103
I. hypsophila	AY641104
I. kirkii	AY641105
I. taiwanensis	AY641106
I. yunguiensis	AY641107
I. sinensis (*I. yunguiensis* type clone)	AY641108
I. sinensis (*I. taiwanensis* type clone)	AY641109